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Studies on the Process of Formation, Nature and Stability of Binding Sites in Molecularly Imprinted Polymers

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ABSTRACT

In Molecular Imprinting the nature of the templated binding sites and the mechanism of their formation are still poorly understood. For this reason our groups are carrying out fundamental studies concerning known imprinting protocols, with the primary aim of shedding light on the role of the template in the different steps of the polymerisation, from the formation of primary chains to the build-up of the porous structure. In this paper we report our initial results concerning copolymers of methacrylic acid (MAA) and ethyleneglycol dimethacrylate (EDMA) and their formation in presence or absence of the templates 9-ethyladenine, ametryn or terbutylazine. Monitoring the monomer disappearance by ^{1}H-NMR showed that the presence of templates such as 9-ethyladenine significantly retarded the polymerisation but otherwise had minor influence on the relative reactivity of the monomers. The latter appeared in most cases to be stoichiometrically incorporated into the polymer. The signals arising from the template experienced little or no shift in the early stage of the polymerisation, although pronounced broadening was observed. By delaying the addition of the template, it was observed that binding sites with high selectivity could be induced more than one hour after the gel point of the system had been passed. Finally, the results of post-polymerisation curing on the dry and swollen state porosities and the recognition properties of terbutylazine imprinted polymers are reported. This treatment when performed at temperatures between 100-120°C, slightly enhanced the selectivity of the polymers, whereas at higher temperatures the polymers lost their molecular recognition properties. Swollen state porosity derived from inverse size exclusion chromatography (ISEC) revealed an interesting sharpening of the pore size distribution for the imprinted compared to the non-imprinted polymers.

INTRODUCTION

Although molecularly imprinted polymers are nowadays well known and increasingly employed in various fields as compound- or group- selective materials,[1] only little effort has been devoted to fundamental characterisation of these materials[2-13]

Molecularly imprinted polymers (MIPs) are typically prepared by polymerisation of commodity monomers (such as methacrylic acid and ethylenglycol dimethacrylate) in the presence of a template.(Figure 1) This results in the formation of binding sites which are complementary in shape and size to the template itself and are able to rebind it selectively in the presence of structurally related analogues. The functional group arrangement is the key to the affinity and selectivity of the templated sites[2, 14] whereas their accessibility and stability affects the performance of the MIPs in dynamic applications such as in chromatography, membrane separation or in chemical sensors.

In non-covalent molecular imprinting this arrangement requires the formation of stable complexes between the template and functional monomers in the pre-polymerisation mixture.[5] These complexes are incorporated in the network during the polymerisation giving rise to imprinted sites differently embedded in the polymer matrix. In addition non-complexed monomer gives rise to non-imprinted sites, thus diluting the selective binding occuring in the templated sites. Previous reports have suggested that the solution structure of the monomer-template complex is preserved during polymerisation and thus reflects the structure of the templated sites.[8, 15, 16] However, apart from indirect structure binding and thermodynamic studies[15, 16] on the finished polymer, there is no direct evidence in support of such a mechanism. Furthermore, it is unclear at what critical monomer conversion or crosslinking density these sites are irreversibly formed and how the template additionally affects the morphology of the materials. Finally, a better understanding of the process of formation of the sites could suggest new techniques to increase site accessibility, eventually leading to materials with improved kinetic properties. On these grounds, our groups decided to carry out a series of fundamental studies concerning known imprinting protocols with the primary aim of investigating the role of the template in the different steps of the polymerisation process, from the formation of the primary chains to the build-up of the porous structure. Ametryn, 9-ethyladenine and terbutylazine (figure 1) were chosen as model templates on account of their strong interactions with methacrylic acid, which have been shown to result in highly selective MIPs.[17, 18]
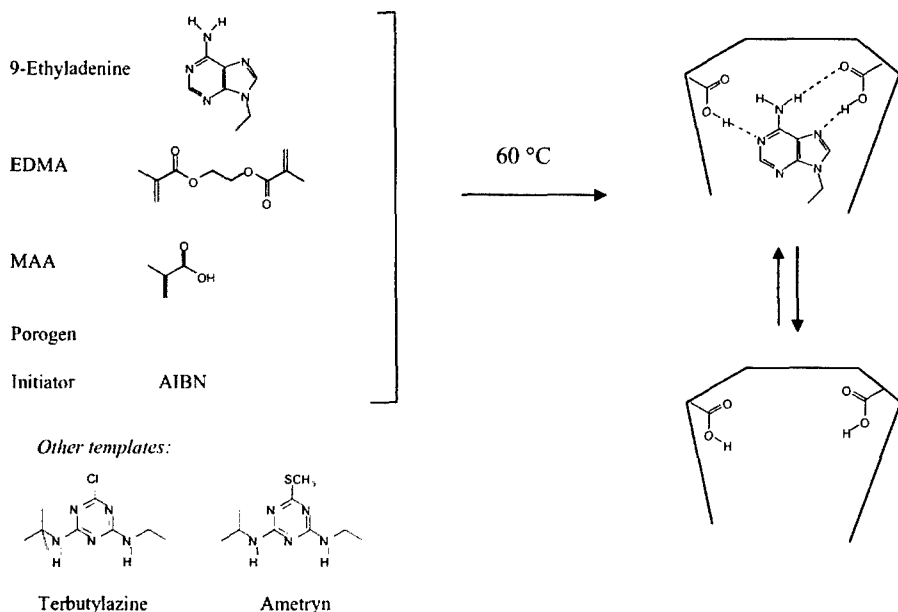

Figure 1. Molecular imprinting of poly(methacrylic acid-*co*-ethyleneglycol dimethacrylate) with the model templates used in the study

EXPERIMENTAL DETAILS

General procedures

All monomers and solvents used for the polymerisation were purified before use. MAA and EDMA were obtained from Sigma-Aldrich Chemie. EDMA was purified by extraction with 10% sodium hydroxide, washing with brine and water, drying over magnesium sulfate followed by distillation. MAA was purified by drying over anhydrous magnesium sulphate followed by distillation under reduced pressure. The porogens (CH_2Cl_2 and $CHCl_3$) were purchased by Sigma-Aldrich Chemie and Acros Chemicals and they were all distilled under a positive nitrogen atmosphere prior to use. AIBN was purchased from Acros Chemicals and recrystallised from methanol before use.

Reagents and solvents used for the *in-situ* NMR experiments were dried beforehand and when mixed they were deoxygenated by purging with oxygen free nitrogen. Polymerisation inhibitors were here removed by elution onto neutral alumina.

The UV lamp used for the photopolymerisations was a high pressure mercury vapour lamp (Philips, HPK 125 W). All chromatographic evaluations were performed using an Agilent 1100 instrument equipped with a binary pump, an autosampler, a variable wavelength detector and a work station. The ^{1}H-NMR spectra were run at Material Research Ireland, Dublin.

The inverse size exclusion measurements were performed at PSS Polymer Standard Services GmbH, Mainz, Germany.

In situ ^{1}H-NMR experiments

All proton nuclear magnetic resonance spectra were recorded on a Bruker DPX-400 (400 MHz) spectrometer. The chemical shifts are reported on the δ scale in parts per million downfield from TMS (0.00 ppm) or with deuterated chloroform as internal reference ($CDCl_3$, 7.26 ppm). Spectra were recorded at 30°C ± 1°C for the characterisation of the individual monomers, template and polymerisation mixtures. For the in-situ monitoring of the polymerisation, MAA (5 mmol) and EDMA (5 mmol) were mixed in an NMR tube and an equal volume of $CDCl_3$ (1.36 ml) was added. The initiator, AIBN, was added (1 % w/w based on the total amount of monomer) and the polymerisation monitored at 60°C for 4 to 7 hours in presence or absence of the template 9-ethyladenine (86.6 mg). At the end of this period the sample appeared solid and no further NMR signal could be detected. The peak areas of the vinyl peaks of MAA and EDMA (figure 2) were used for the calculation of the curing profiles reported in Figure 3. The cure profiles were obtained by plotting the curing time in minutes against the component integral peak area in percent. The signals of the protons in position 2 and 8 of the 9-ethyladenine were also monitored during the polymerisation as shown in Figure 2(A).

Late addition of the template

The preparation of MIPs and NIPs (Non Imprinted Polymers) on a small scale (500 mg of raw polymer) and their *in-situ* testing by batch rebinding experiments was performed as described elsewhere.[19] 20 ml scintillation vials were used as polymerisation reactors. A mother solution was prepared by mixing 11.4 ml EDMA (12 mmol), 1,02 ml MAA(12 mmol) 18 mg ABDV and 12 ml $CHCl_3$. A template solution was prepared by dissolving 1.42 g ametryn in 10 ml $CHCl_3$. 1 ml of the mother solution was dispensed into each vial; the vials were purged with nitrogen for 5 min and then placed in a thermostatted bath at 40°C. Pairs of vials were

removed at different times after the start of the polymerisation and cooled down on ice for 5 minutes. To one of these, pure solvent was added (so that a NIP was obtained upon polymerisation) while to the other the concentrated template solution was added (200μl in both cases). After addition they were manually shaken and left on ice for an additional 10 minutes. Thereafter the vials were returned in the thermostat bath and further polymerised for additional 24 hours.

After the polymerisation the polymers were subjected to different solutions to study the release and rebinding of the template.[19] The porogen chloroform was used for the release tests, $CH_3OH/CH_3COOH/H_2O$ 60/30/10 (v/v/v) to wash the polymers and a solution of ametryn in the porogen (0.5 mM in $CHCl_3$) was used for the rebinding tests. The concentration of the free template in solution was determined after 48 hours by reversed phase HPLC-UV (Luna ODS column by Phenomenex, CH_3CN/NH_4OAc 40 mM 75/25 as mobile phase, 1 ml/min flow rate, wavelength of detection 254 nm). The amount of template bound to MIPs and NIPs was calculated assuming a 500 mg weight of polymer in each vial.

Curing experiments

Terbutylazine-imprinted polymers were prepared according to a previously reported procedure.[19] EDMA (20 mmol, 3.8 ml), MAA (4 mmol, 0.34 ml) terbutylazine (1mmol), AIBN (0.24 mmol, 40 mg) were dissolved in 5.6 ml of dichloromethane and the solution was then transferred to a glass tube (14 mm i.d.). The polymerisation mixture was degassed with nitrogen for 5 min while cooled on ice and then the tubes were flame sealed. All the tubes were placed at *ca.* 10 cm distance from the UV light source and then turned at regular intervals during the first 30 min, to obtain a more even exposure. After 24 hrs irradiation the tubes were cured for an additional 24 hours at elevated temperatures. The tubes were then crushed, the polymers were ground and sieved under water and the particle size fraction 25-36 μm was collected. This fraction was then repeatedly washed with 50 ml aliquots of CH_3OH/H_2O 1/1, CH_3OH, CH_3OH/CH_3COOH 80/20 and CH_3OH and then used for the chromatographic evaluation and the pore analysis.

The 25-36 μm particle fraction of each polymer batch was slurry packed into stainless steel HPLC columns (125 x 4 mm) using MeOH/H_2O 80/20 as pushing solvent at pressures up to 300 bar. The columns were then evaluated using $CH_3CN/H2O/CH_3COOH$ 92.5/2.5/5 or CH_3CN as mobile phase, at a flow rate of 1 ml/min. The wavelength of detection was 254 nm and the injection volume was 10 μl. 1 mM solutions of the analytes in the mobile phase were injected unless otherwise stated. The capacity factor k′ was calculated as $(t-t_0)/t_0$ where t is the retention time of the analyte and t_0 the retention time of a void marker (acetone)

Inverse Size Exclusion Chromatography

The previously described columns were used for the inverse size exclusion measurements. The measurements were performed using an Agilent 1100 HPLC system comprising a binary pump, an autosampler and a variable wavelength detector. THF was used as mobile phase at a flow rate of 0.2 ml/min. The wavelength of detection was again 254 nm. 1 mg/ml solutions of polystyrene standards of different molecular weights were in this case injected for the evaluation. Acetone was used as void marker.

The pore analysis based on the inverse size exlusion measurements was carried out by means of the PSS Porocheck Software. The pore size distributions were calculated as average on the volume.

Pore analysis (N$_2$ adsorption porosimetry)

Pore and surface area analysis were performed using a Quantachrome Autosorb 6B (Quantachrome Corporation, Boynton Beach, FL, USA). A sample of polymer (50 mg) was degassed at room temperature overnight under vacuum. The adsorption and desorption isotherms were then recorded using an 80-point pressure table and 15 s equilibration time.
Surface areas, pore volumes and pore size distributions were determined by means of the Autosorb Software for Windows version 1.11. The surface areas were determined using the BET model, pore volumes and pore size distributions using the BJH model.

DISCUSSION

Our initial aim was to gain knowledge about the initial stages of the thermal copolymerisation of MAA and EDMA in presence or absence of the template 9-ethyladenine. This template is known to interact strongly with carboxylic acids in aprotic solvents[20] and a significant part should exist in the complexed form prior to polymerisation, as indicated in Figure 1. Complexation was supported by pronounced shifts observed for the exocyclic amine and aromatic ^{1}H-NMR signals of the template upon addition of MAA (NH$_2$: -2,4 ppm; H$_2$: +0,1 ppm; H$_8$: -0,1 ppm). These signals appear in a region of the spectrum which is free from any other interfering signals arising from the monomers (figure 2). *In-situ* ^{1}H-NMR thus allowed the characteristic protons of the template as well as the vinyl protons of MAA and EDMA, to be monitored during the initial stage of the polymerisation (figure 2).
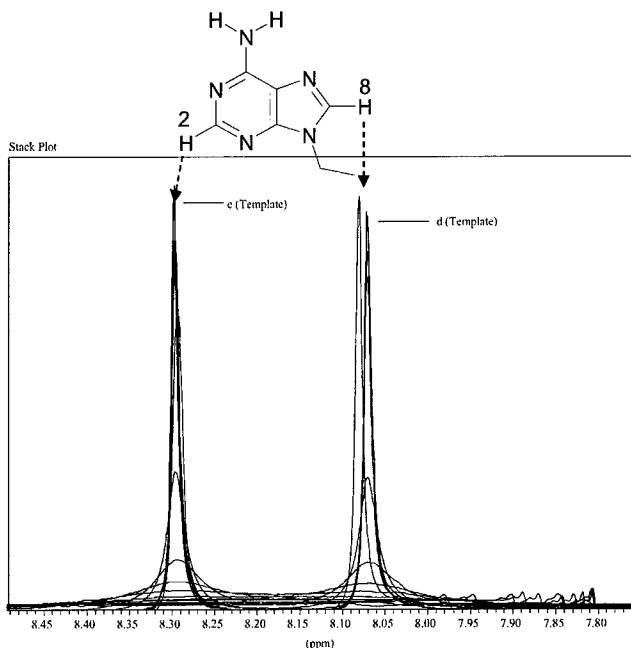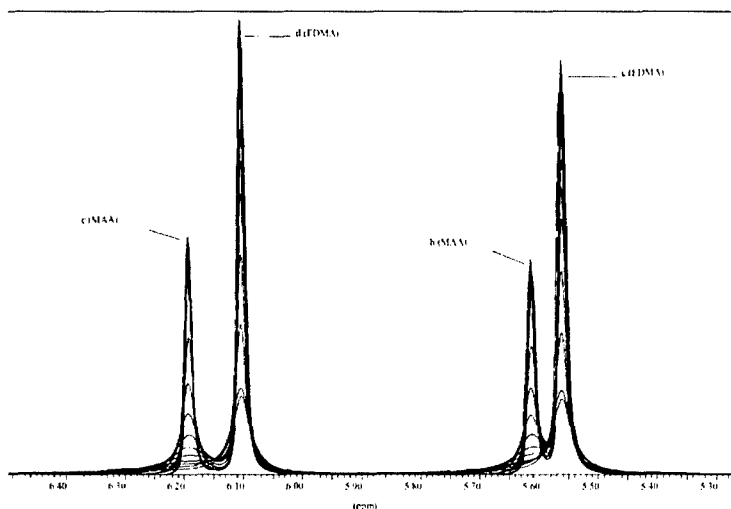

Diagram A

Diagram B

Figure 2. Characteristic proton signals of 9-ethyladenine (A) and the monomers (B) during the imprinting process

Concerning the template signals a slight upfield peak shift (0.01ppm) for H_8 was observed during the first 3 minutes of the polymerization. After that no significant peak shift could be observed. This indicates that the degree of monomer-template complexation is constant in this interval. The signals then experienced broadening early on during the polymerisation. Interestingly this broadening was more pronounced for the template than for the monomer related signals, indicating that the template mobility becomes severely restricted. Figure 3 shows the integral areas of the relevant signals plotted versus the time of polymerisation.
Integrals of the monomer signals could be obtained during the first two hours of the polymerisation. After this point pronounced broadening due to polymerisation occured. Thus, the integrals of these signals cannot be taken as a quantitative measure of the absolute conversion of the monomers. However, they do reflect the absolute and relative monomer reactivities during the initial polymerisation phase. In view of the relative initial slopes of the rate curves, the monomers appeared, in most cases, to be stoichiometrically incorporated in the polymer.
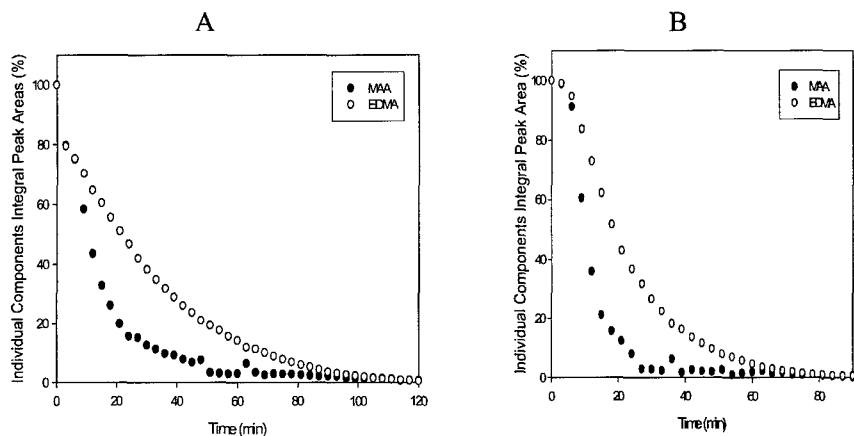
Figure 3. Integral areas from Figure 2 of the vinyl protons of MAA and EDMA as a function of polymerisation time in the absence (A) and in the presence (B) of the template (MAA/EDMA/9-ethyladenine: 5/5/1 in CDCl₃).

However, comparing the kinetics in the presence (figure 3, B) and absence of template (figure 3, A), a retardation of the onset of polymerisation was seen in the presence of template. After an initial lag time the monomers are converted at a rate that appears slightly faster than in absence of template. The origin of this phenomenon and its implication on the mechanism of site formation is still unclear. However it must be noted that the reactivity of MAA in free radical homopolymerisation depends on the extent and types of complexes present.[21] Further experiments are being performed which will be the topic of a forthcoming publication.[22], in particular different compositions of the polymerisation mixtures and different templates have been studied: in all cases the presence of the template significantly retarded the polymerisation.

The question arose with regards to what point in time the templated sites are irreversibly formed by crosslinking. To answer this question, another series of experiments was performed by adding the template after the start of the polymerisation. In order to minimise the dilution of the system upon each addition, this experiment required the use of a template that is highly soluble in the porogenic solvent. For this purpose the triazine ametryn was used. The polymers were prepared in standard scintillation vials (500 mg) by thermal initiation (ABDV, 40°C) and then tested *in situ* by studying the release and rebinding of the template under equilibrium conditions. The amount of template rebound to the MIP and to the NIP for the thermally initiated MIPs and NIPs are shown in figure 4.
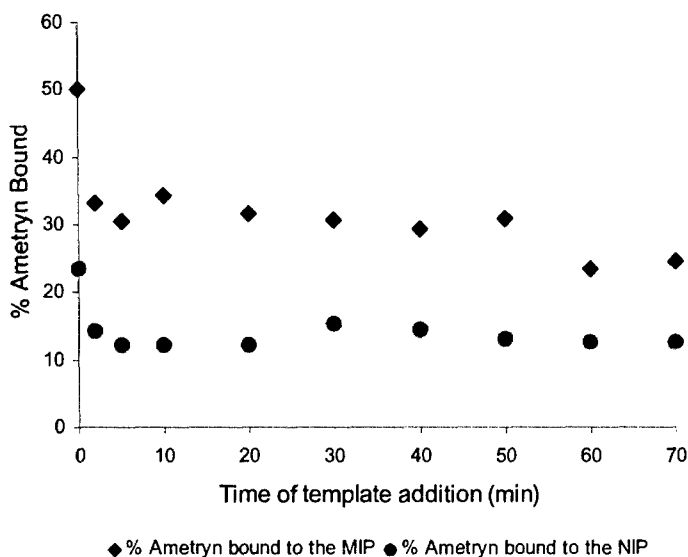
Figure 4. Percentage of template rebound in an equilibrium batch rebinding experiment performed on MIPs (diamonds) and NIPs (circles) prepared by delayed addition of template (for the conditions of the equilibrium batch rebinding see the Experimental details).

A pronounced imprinting effect was seen for all the materials prepared within the first hour after start of the polymerisation. Although the reference materials (t=0) adsorbed somewhat higher amounts of template (possibly related to radical quenching in the manipulated samples), the selectivity changed only slightly in this interval and started to decrease only after 60 minutes. Thus, templated sites exhibiting similar selectivity to that of the native material can be induced well after the gel point of the system has been passed (typically less than 30 minutes). These results may thus reveal the monomer conversion and critical crosslinking level required to stabilise the templated sites. Such studies are presently being performed.[22]

Given that templated sites can be induced well after the start of the polymerisation, the question arose of to what extent these sites collapse upon subsequent removal of the template and the thermal stability of the sites. In view of the large portion of unreacted double bonds present in typical MIPs, could the sites be further stabilised by post-treatment at higher temperatures in the presence of the template ? In an attempt to answer these questions MIPs and NIPs were prepared by photochemical initiation at 15°C in flame sealed vials which were subsequently cured for an additional 24 hrs at elevated temperatures between 80°C and 160°C . The materials were then worked up and evaluated as stationary phases in chromatography. Figure 5 shows the capacity factors of the template (terbutylazine) on the imprinted and non-imprinted polymers versus the temperature of curing.
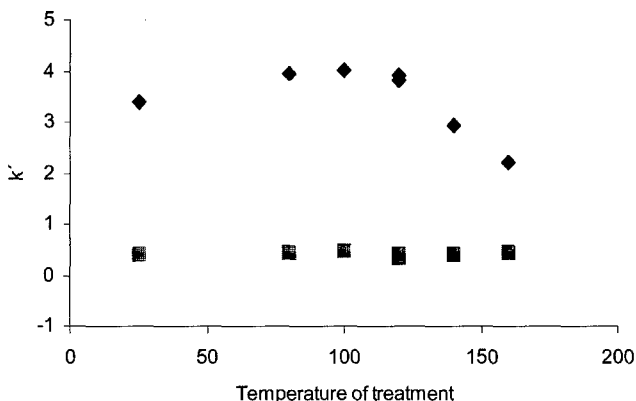
Figure 5. Capacity factors of terbutylazine (10µl of a 1mM solution in the mobile phase CH$_3$CN/CH$_3$COOH/H$_2$O 92.5/5/2.5) on columns packed with MIPs (diamonds) and NIPs (squares) cured at different temperatures.

A slight but significant increase in the retention is observed on the MIP when the curing temperature is increased up to 120°C. This behaviour is absent on the NIP and seems therefore to be related to the templated sites. Curing at higher temperatures leads to a loss in selectivity, very similar to our previous results from annealing experiments.[10] Interestingly the temperature interval where the decrease occurs agrees roughly with the glass transition temperature for linear poly(methylmethacrylate). Thus it may due to large conformational changes in less crosslinked regions of the network.

The polymers prepared using chloroform or dichloromethane are gel like and exhibit no porosity in the dry state.[9] Thus nitrogen sorption does not reveal any effect of the presence of template or curing on the pore size distributions and morphologies of the materials. For this purpose the porosity in the swollen state needs to be investigated. This can be done by inverse size exclusion chromatography (ISEC), providing the exclusion volume for polystyrene standards of known molecular radii. From the exclusion volumes, the accessible pore volume versus molecular radius can be modelled. As seen in figure 6, the polymers prepared using dichloromethane as porogen exhibit a large volume of pores with a diameter between 2 and 10 nm.
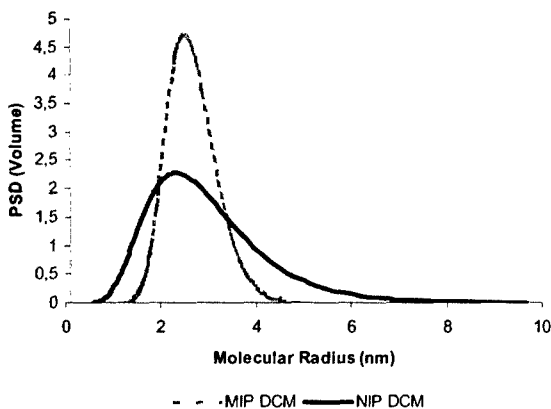
Figure 6. Pore size distributions obtained from ISEC measurements on an ametryn imprinted polymer (dotted curve) or non imprinted polymer (continuous curve) prepared using dichloromethane as porogen. The polymers were non porous in the dry state but gave a surface areas of 930 m^2/cm^3 (NIP) and 810 m^2/cm^3 (MIP) in the ISEC measurement.

This gives rise to a very high surface area for these materials of nearly 1000m^2/mL. The presence of template leads to a pronounced sharpening of the pore size distribution. The origin of this effect is unclear but the analogy with the role of templates in the synthesis of mesoporous materials is striking.[23]

CONCLUSIONS

The first studies on the formation and the nature of templated sites have shown this to be a slow process and that high affinity binding sites can be induced by delaying the addition of template. This may have practical implications concerning template bleeding and the kinetic properties of the materials. Thus template added at later stages is expected to be less embedded and to result in more accessible binding sites. From these sites it should also be easier to remove the template quantitatively. Curing of the materials at elevated temperatures up to 120°C seems to stabilise some sites and make them more accessible, whereas curing at even higher temperatures leads to destruction of sites.

ACKNOWLEDGEMENTS

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